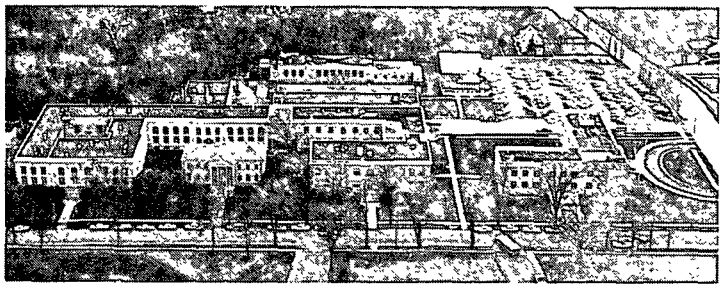

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BIOCHEMICAL CHANGES ASSOCIATED WITH ZYGOTIC PINE EMBRYO DEVELOPMENT

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Biochemical Changes Associated With Zygotic Pine Embryo Development

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ABSTRACT

Periodically from prefertilization to near maturation, pine (Pinus resinosa Ait. and Pinus strobus L.) ovules were analyzed for several biochemical constituents, and the results were expressed on a fresh weight basis. Lipid accumulated in parallel with the growth of the developing seeds. Soluble protein also accumulated but only in the initial stages of development. ATP content peaked approximately two weeks after fertilization, followed about one week later by the energy charge; these peaks were associated with maximal growth stages of the developing embryos. Likewise, peaks of glutathione (GSH) and ascorbic acid (AA), two water soluble reductants, preceded or coincided with the ATP maximum. At late stages of seed development, dissection of the more mature ovules into embryos and gametophytes for analysis revealed that most of the ATP, GSH, and AA was associated with the embryonic tissue. On the other hand, this segregation showed that virtually no proanthocyanidin was located in the developing embryos proper, although they contained other reductants, some of which were probably phenolics. Also, general staining with reagents for phenolics and thiols indicated that the former occurred primarily in

the developing seed coat, whereas GSH was in the embryo per se. These findings are consistent with roles for ATP, GSH, and AA in the growth and development of zygotic pine embryos; however, it would appear that lipid and protein are being stored for subsequent germination events and that much of the phenolic component is segregated from the developing embryo.

Key words - Pine, embryogenesis, biochemistry.

INTRODUCTION

With the exception of recent research in the molecular biology area (Misra and Bewley, 1985; Aspart, Meyer, Laroche, and Penon, 1984; Weidner and Wielgat, 1984), biochemical investigations of zygotic seed development still seem to be uncommon, especially in gymnosperms. This appears to be due partly to the minute quantities of sample available, particularly in early stages of the process. We have undertaken research of this nature in order to better understand what might be happening biochemically in conifer somatic embryogenesis occurring in vitro, granting that the two processes may not be completely identical. Control of the latter process is expected to further our efforts to enhance the value of forest resources and accelerate mass propagation of desirable tree specimens. The behavior of cell suspension cultures relative to the results presented here will be treated elsewhere.

METHODS AND MATERIALS

Developing seed ovules were removed manually from cones collected periodically from pine trees growing in the vicinity of Appleton,

Wisconsin, in the summers of 1981-1984 (most of the data in this report originated in 1982 and 1984). The species monitored were red pine (Pinus resinosa Ait.) and white pine (Pinus strobus L.). Based upon microscopically observed zygotic embryo development, in all cases reported here fertilization occurred at the end of June or the beginning of July.

Freshly picked cones were brought to the laboratory and processed immediately to remove the ovules. Until late July or August the developing embryos were too small to make separation of embryonic and gametophytic tissue practical; however, this separation was carried out in later stages of development, whereupon the tissues were analyzed separately. All ovules, immature embryos, and gametophytes were frozen briefly (24 to 48 h) in extracting solvent before analysis for GSH, reductants, and proanthocyanidins. The ovule protein determination was conducted on samples frozen for up to 2.5 months. All other analyses employed fresh samples.

Total lipids were determined simply by a gravimetric procedure following extraction with a nonpolar solvent system (Bligh and Dyer, 1959). AA was extracted with a metaphosphoric acid/acetic acid solvent and measured fluorimetrically after conversion to dehydroascorbic acid and reaction with o-phenylenediamine (Udenfriend, 1969). Protein was determined with the Bradford reagent (Bradford, 1976) after extraction with 10 mol m^{-3} HEPES, pH 7.5, or 5 mol m^{-3} potassium phosphate buffer, pH 6.8, as indicated. GSH was extracted with 70% acetone (v/v) and measured by

its formation of a disulfide with 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) to liberate the thionitrobenzoate dianion absorbing at 412 nm (Jocelyn, 1962). Aliquots of the same extracts were assayed for total reductants and "soluble" proanthocyanidins. Proanthocyanidins (condensed tannins), soluble or insoluble in 70% acetone, were estimated using the hot butanol/HCl technique as employed by Stafford and Cheng (1980). The standard curve was prepared using a "Karchesy fraction" isolated from Douglas-fir callus by Monroe (Monroe and Johnson, 1984). Total reductants were estimated with the ferric chloride-potassium ferricyanide reagent, proposed to measure total phenolics (Singh, Singh, and Sanwal, 1978), using an arbitrary catechin standard. ATP, ADP, and AMP were all analyzed by the luciferin-luciferase photoluminescence technique (Strehler and McElroy, 1957) after extraction with DMSO. The energy charge was computed using the formula developed by Atkinson (Atkinson, 1969).

All analyses were conducted on pooled samples of ovules, immature embryos, or gametophytes of a given collection date. All assay procedures consisted of at least triplicate determinations, except for the lipid and "insoluble" proanthocyanidin analyses, which were not routinely replicated. For the GSH, reductants, and "soluble" proanthocyanidin analyses, triplicate aliquots of an extract of a pooled sample were assayed. For the AA and protein determinations, replication consisted of separate extracts of three samples of pooled material. For ATP and energy charge analyses, triplicate assays were conducted on separate extracts

of two samples of pooled material; in this case, one of the two samples was always double the size of the other, e.g., 10 ovules and 5 ovules, etc. Where superscript letters appear with graphed means, the data have been subjected to ANOVA followed by Duncan's new multiple range test; means of individual plots with common letters are not significantly different from one another at the 95% confidence level.

RESULTS

Lipid accumulation in ovules of both pine species was plotted as a common curve as shown in Fig. 1F, which also contains some protein data for red pine (not determined for white pine). Rapid lipid accumulation began shortly after fertilization and appeared to be tapering off by the end of August. Soluble protein accumulation in red pine ovules exhibited a similar trend but ceased much earlier, with the bulk of it building up in embryos (not shown but observed when fresh embryos and gametophytes were extracted with the phosphate buffer and compared). The GSH content of the ovules of both species passed through a maximum in mid-July (Fig. 1C). As also shown in Fig. 1C, most of this GSH was associated with the embryo rather than with the gametophyte during the month of August in the case of white pine; presumably, this was also the distribution during July when the sample size precluded separate analysis. Reaction of ovules with the DTNB reagent for thiols resulted in a yellow stain that seemed exclusively located in the embryo as viewed under a binocular microscope. The AA content of developing

red pine ovules also passed through a maximum in mid-July, again with most of it in the embryo to the extent that late distributions can be extrapolated back to July (Fig. 1D). The total reductants analysis, which includes at least GSH, AA, and phenolics, reflected the mid-July peaks of GSH and AA, but it also revealed a second peak in August (Fig. 1E) thought to consist mainly of phenolics in the developing seed coat as indicated by staining with the reagent and proanthocyanidin distributions (below).

(Fig. 1 here)

ATP content of developing red pine ovules also peaked in mid-July as shown in Fig. 1A with much of it apparently confined to the embryos. However, the energy charge value peaked extremely high about one week later (Fig. 1B) and seemed to be declining at a slower pace than the ATP content per se. Furthermore, the measured energy charge in gametophytic tissue was substantial even though the ATP content was relatively low. It is quite apparent that fertilization places increased demands on energy generation for development. This is seen in the substantial increases in both ATP and energy charge beginning in early July.

Determination of the proanthocyanidin content of developing ovules resulted in an erratic picture, with spot checks of biological replication indicating that it was very poor; nevertheless, analyses of gametophytic and embryonic tissues in white pine in August indicated that none of the "soluble" and very little of the "insoluble" proanthocyanidins were located in the embryos (not shown).

Observed changes in the mean fresh weight of pooled samples of developing ovules of white pine are shown in Fig. 2. Note what appears to be a brief lag phase in early July following fertilization.

(Fig. 2 here)

DISCUSSION

Many of the same biochemical parameters investigated here in developing zygotic pine ovules were studied in developing rape seeds by Ching and Crane (1974) with nearly the same results. For example, both varieties of developing rape seed used in their study yielded curves for lipid and protein accumulation similar to those in Fig. 1F. Likewise, they observed an ATP peak about 5 weeks after anthesis, whereas the ATP maximum appeared in red pine ovules between two and three weeks postfertilization (Note: fertilization is delayed one year after pollination in these pines). Energy charge peaks in the rape seed study also lagged behind peaks in ATP content in some instances but did not reach quite as high as the extreme value of about 0.98 for red pine ovules on July 24. These ATP, energy charge and lipid results are to be expected to meet the short term and long term energy requirements of the developing and mature seed, respectively. Interpretation of the protein accumulation must await qualitative information on the specific protein(s) involved. A similar study was conducted on sorghum seed development by Subramanian (1983) but did not include ATP. There have been some investigations on developing

seeds that have focused on specific lipids and proteins, e.g., studies by Wright, Park, Leopold, Hasegawa, and Janick (1982) on Theobroma cacao and by Stinessen, Peumans, and DeLange (1984) on developing rice embryos.

The mid-July maximum in ATP content coinciding with maxima in AA and GSH content (Fig. 1A,C,D) is consistent with the findings from histochemical studies on zygotic embryogenesis in Limnophyton obtusifolium (Shah and Pandey, 1978). Staining for AA and sulfhydryl groups in that case was most intense at the first appearance of globular embryos with attached suspensors. This roughly corresponds to the mid-July stage after developing conifer proembryos have reached the bottom of the erosion zone in the gametophyte and have started their period of maximum growth. There has been a histochemical study of pine (Pinus sylvestris) ovular development published (Franssen-Verheijen and Willemse, 1982), but observations ceased shortly after fertilization, making them of limited relevance to the present study. The role of ascorbic acid in plant development is still largely speculative (Chinoy, 1984; Joshi, Sharma, Rathore, Vaishnov, and Singh, 1980), although there would appear to be some evidence (Dhar, Patel, and Shah, 1980) for roles in both cell division and elongation. Actually, ascorbic acid has been associated with plant embryogenesis almost since its discovery (Bonner and Bonner, 1938) and at one point was under consideration as a growth hormone (Tonzig and Marre, 1961).

GSH has been tied to cell division events rather than to elongation. A role for GSH in somatic embryogenesis in wild carrot was demonstrated recently (Earnshaw and Johnson, 1985), and similar data exist for AA in that system (Earnshaw, 1986). The observed decreases in GSH and AA concentrations as wild carrot somatic embryogenesis proceeds may correspond to the decline in both GSH and AA seen after mid-July in the zygotic pine embryo development here. There is growing evidence that GSH is involved in the control of development in organisms other than higher plants also. Of particular note are recent reports on a role for GSH in slime mold development (Allen, Farmer, Toy, Newton, Sohal, and Nations, 1985). Seasonal variations of both the GSH and AA contents of conifer leaves have been reported (Esterbauer and Grill, 1978; Esterbauer, Grill, and Welt, 1980). Because the pentose phosphate pathway provides reducing equivalents for these antioxidants (GSH and AA), the histochemical study of Georgieva (1982) on Lilium seed development is also relevant. Since the NADPH produced in the pentose phosphate pathway is the reducing agent in the enzymatic conversion of GSSG to GSH via GSSG reductase, it follows that peaks in GSH and in the activity of this pathway might occur together.

Although no such evidence was forthcoming in this investigation, roles for phenolic compounds in developmental processes were proposed long ago (Reed, 1949; Van Fleet, 1954). Phenolic acids in stratifying sugar pine seeds were examined recently by Noland and Murphy (1981a, 1981b) but seemed to have little function even

in dormancy. Tannins are usually viewed as metabolic endproducts serving to deter predators, but this may not be entirely true. Their solubility behavior here suggests a heterogeneous group; lumping them together for biological purposes may be as hazardous as is the case for other "phenolics."

At early stages of development the small ovule sizes mandated pooled samples which were therefore employed throughout. It should be recognized that biological variation was taken into account by this pooling and that the statistics appearing with the plots presented address primarily extraction and analysis variability. Although not shown, some of the data presented here have also been expressed on a per ovule basis with little change in interpretation, although some differences were evident in the various plots, particularly in late stages when fewer developing seeds made up the analyzed sample weight.

Some advances on the biochemistry of seed development are occurring through studies in which zygotic embryos are cultured in vitro (Monnier, 1980; Hsu, Bennett, and Spanswick, 1984). Other biochemical progress stems from enzyme studies which seem to be concentrated largely on mainline carbon and nitrogen metabolism (Murray and Kennedy, 1980; DeKlerk, 1984; Bhambri, 1982; Hildebrand, 1983), hormone effects (Ackerson, 1984; Brinegar, Stevens, and Fox, 1985; Finkelstein, Tenbarge, and Crouch, 1985; Prevost and LePage-Degivry, 1985), and organelle function (Kolloffel, 1983).

With a few notable exceptions (Ermakov, Barantseva, Matveeva, and Korolev, 1981; Yermakov, Barantseva, and Matveeva, 1981; Franssen-Verheijen and Willemse, 1982; Johnson and Carlson, 1982 Raghavan, 1976; Ryczkowski and Ryczkowska, 1973), the pace has not quickened greatly yet for biochemical studies of seed development in gymnosperms. Since observations of somatic embryogenesis in plant tissue cultures have become more commonplace recently, biochemical studies, including some from this laboratory (Feirer, Mignon, and Litvay, 1984; Earnshaw and Johnson, 1985), often involve these more convenient systems. Analytical data such as presented in this report will eventually be integrated with our metabolic studies on somatic embryo development to provide a more complete picture of conifer seed development.

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LITERATURE CITED

Ackerson, R. C., 1984. Regulation of soybean embryogenesis by abscisic acid. Journal of Experimental Botany, 35, 403-13.

Allen, R. G., Farmer, K. J., Toy, P. L., Newton, R. K., Sohal, R. S., and Nations, C., 1985. Involvement of glutathione in the differentiation of the slime mold, Physarum polycephalum. Development, Growth & Differentiation, 27, 615-20.

Aspart, L., Meyer, Y., Laroche, M., and Penon, P., 1984. Developmental regulation of the synthesis of proteins encoded by stored mRNA in radish embryos. Plant Physiology, 76, 664-73.

Atkinson, D., 1969. Regulation of enzyme function. Annual Review of Microbiology, **23**, 47-68.

Bligh, E. G., and Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology, **37**, 911-17.

Bonner, J., and Bonner, D., 1938. Ascorbic acid and the growth of plant embryos. Proceedings of the National Academy of Sciences, U.S.A., **24**, 70-5.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry, **72**, 248-54.

Brinegar, A. C., Stevens, A., and Fox, J. E., 1985. Biosynthesis and degradation of a wheat embryo cytokinin-binding protein during embryogenesis and germination. Plant Physiology, **79**, 706-10.

Ching, T. M., and Crane, J. M., 1974. Adenylate energy pool and energy charge in maturing rape seeds. Plant Physiology, **54**, 748-51.

Chinoy, J. J. (ed N. J. Chinoy)., 1984. The role of ascorbic acid in growth, differentiation and metabolism of plants. The Hague, Martinus Nijhoff/Dr. W. Junk.

DeKlerk, G. J., 1984. Changes in protein synthesis during the development of Agrostemma githago seeds. Physiologia Plantarum, 62, 607-14.

Dhar, A. C., Patel, K. R., and Shah, C. K., 1980. Role of ascorbic acid in auxin induced cell elongation - a cytochemical study. Histochemistry, **69**, 101-9.

Earnshaw, B. A., and Johnson, M. A., 1985. The effect of glutathione on development in wild carrot suspension cultures. Biochemical and Biophysical Research Communications, **133**, 988-93.

Earnshaw, B. A., 1986. The role of cellular antioxidants (glutathione and ascorbic acid) in the growth and development of wild carrot suspension cultures. Ph.D. Thesis. The Institute of Paper Chemistry, Appleton, WI, U.S.A.

Ermakov, I. P., Barantseva, L. M., Matveeva, N. P., and Korolev, Yu. N., 1981. Study of cell growth and protein and RNA metabolism during development of the archegonium in Pinus siberica Du Tour. Biologicheskie Nauki (Moscow), **2**, 88-92. (Chemical Abstracts **95** 39186b).

Esterbauer, H., and Grill, D., 1978. Seasonal variation of glutathione and glutathione reductase in needles of Picea abies. Plant Physiology, **61**, 119-21.

Esterbauer, H., Grill, D., and Welt, R., 1980. Der jahreszeitliche rhythmus des ascorbinsauresystems in nadeln von Picea abies. Zeitschrift fur Pflanzenphysiologie, **98**, 393-402.

Feirer, R., Mignon, G., and Litvay, J. D., 1984. Arginine decarboxylase and polyamines required for embryogenesis in wild carrot. Science, **223**, 1433-5.

Finkelstein, R. R., Tenbarge, K. M., and Crouch, M. L., 1985. Role of ABA in maturation of rapeseed embryos. Plant Physiology, **78**, 630-6.

Franssen-Verheijen, M. A. W., and Willemse, M. T. M., 1982. Histochemical study of ovular development in Pinus sylvestris. Phytomorphology, **32**, 345-63.

Georgieva, I. D., 1982. Histochemical study of pentose phosphate pathway dehydrogenases during embryo and endosperm development of Lilium regale. Phytomorphology, 32, 381-5.

Hsu, F. C., Bennett, A. B., and Spanswick, R. M., 1984. Concentrations of sucrose and nitrogenous compounds in the apoplast of developing soybean seed coats and embryos. Plant Physiology, 75, 181-6.

Jocelyn, P. C., 1962. The effect of glutathione on protein sulfhydryl groups in rat liver homogenates. Biochemical Journal, 85, 480-5.

Johnson, M. A., and Carlson, J. A., 1982. Some redox considerations in conifer tissue culture. In ed. A. Fujiwara Proceedings of the Fifth International Congress of Plant Tissue and Cell Culture, Tokyo, Japanese Association for Plant Tissue and Cell Culture, Pp. 221-2.

Joshi, A. K., Sharma, N. S., Rathore, K. S., Vaishnov, P. P., and Singh, Y. D., 1980. Auxin oxidizing systems and ascorbic acid turnover in relation to physiology of dwarfism in Sorghum bicolor L. Moench. Biochemie und Physiologie der Pflanzen, 175, 208-15.

Kolloffel, C., and Matthews, S., 1983. Respiratory activity in pea cotyledons during seed development. Journal of Experimental Botany, 34, 1026-36.

Misra, S., and Bewley, J. D., 1985. The messenger RNA population in the embryonic axes of Phaseolus vulgaris during development and following germination. Journal of Experimental Botany, 36, 1644-52.

Monnier, M., 1980. Contribution de la culture de l'embryon zygotique a la connaissance du developpement et de la physiologie de l'embryon in situ. Bulletin de la Societe Botanique de France. Actualities Botaniques, **127**, 59-70.

Monroe, S. H., and Johnson, M. A., 1984. Membrane-bound O-methyltransferase of Douglas-fir callus. Phytochemistry, **23**, 1541-3.

Murphy, J. B., and Noland, T. L., 1981a. Changes in phenolic acids and abscisic acid in sugar pine seed coats during stratification. Physiologia Plantarum, **52**, 370-4.

Murphy, J. B., and Noland, T. L., 1981b. Changes in phenolic acids and abscisic acid in sugar pine embryos and megagametophytes during stratification. Physiologia Plantarum, **52**, 375-9.

Murray, D. R., and Kennedy, I. R., 1980. Changes in activities of enzymes of nitrogen metabolism in seedcoats and cotyledons during embryo development in pea seeds. Plant Physiology, **66**, 782-6.

Prevost, I., and LePage-Degivry, M. T., 1985. Changes in ABA content in axes and cotyledons of developing Phaseolus vulgaris embryos and their physiological consequences. Journal of Experimental Botany, **36**, 1900-5.

Raghavan, V., 1976. Experimental embryogenesis in vascular plants. London, Academic Press. p. 88.

Reed, H. S., 1949. The action of quinones on mitosis. Experientia, **5**, 237-9.

Ryczkowski, M., and Ryczkowska, H., 1973. Free amino acids in the environment of the developing embryo during exponential phase of its growth (mono- and dicotyledonous plants). Bulletin de L'Academie Polonaise des Sciences , 21, 759-66.

Shah, C. K., and Pandey, S. N., 1978. Histochemical studies during embryogenesis in Limnophyton obtusifolium. Phytomorphology, 28, 31-42.

Singh, M., Singh, S. S., and Sanwal, G. G., 1978. A new colorimetric method for the determination of phenolics. Indian Journal of Experimental Biology, 16, 712-14.

Stafford, H. A., and Cheng, T.-Y., 1980. The procyanidins of Douglas-fir seedlings, callus, and cell suspension cultures derived from cotyledons. Phytochemistry, 19, 131-5.

Stinissen, H. M., Peumans, W. J., and DeLange, E., 1984. Abscisic acid promotes lectin biosynthesis in developing and germinating rice embryos. Plant Cell Reports, 3, 55-9.

Strehler, B. L., and McElroy, W. D., 1957. Assay of adenosine triphosphate. Methods in Enzymology, 3, 871-3.

Subramanian, V., 1983. Biochemical changes in seed development of sorghum. Phytochemistry, 22, 1097.

Tonzig, S., and Marre, E., 1961. Ascorbic acid as a growth hormone. In ed. R.M. Klein Plant Growth Regulation, Ames, Iowa State University Press. p. 725-34.

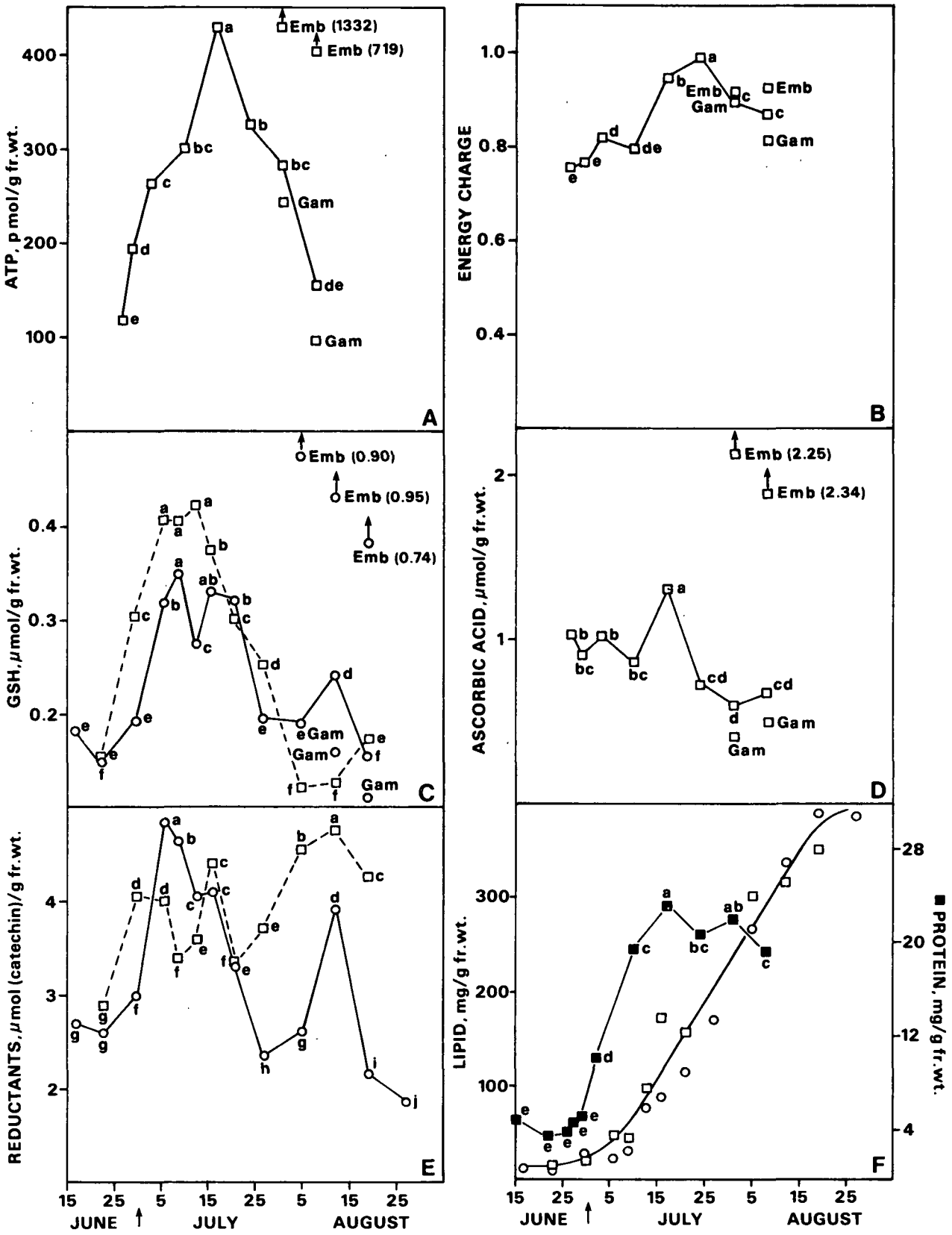
Udenfriend, S., 1969. Fluorescence assay in biology and medicine, Vol. II, New York, Academic Press. pp. 321-2.

Van Fleet, D. S. 1954. The significance of histochemical localization of quinones in the differentiation of plant tissues. Phytomorphology, 4, 300-10.

Weidner, S., and Wielgat, B., 1984. Transcriptional activity of chromatin in developing and germinating triticale embryos. Physiologia Plantarum, **60**, 139-42.

Wright, D. C., Park, W. D., Leopold, N. R., Hasegawa, P. M., and Janick, J., 1982. Accumulation of lipids, proteins, alkaloids, and anthocyanins during embryo development in vivo of Theobroma cacao L. Journal of the American Oil Chemists Society, **59**, 475-9.

Yermakov, I. P., Barantseva, L. M., and Matveeva, N. P., 1981. Cytochemical study of DNA during egg cell maturation and early embryogenesis in Pinus sibirica du Tour. Ontogenez, **12**, 339-45. (Chemical Abstracts, **95**, 111868v).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Emb (719)
Gam
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ENERGY CHARGE
Emb
Gam
B
GSH, μmol/g fr.wt.
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Emb (0.95)
Emb (0.74)
Gam
C
ASCORBIC ACID, μmol/g fr.wt.
Emb (2.25)
Emb (2.34)
Gam
D
REDUCTANTS, μmol (catechin)/g fr.wt.
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JUNE
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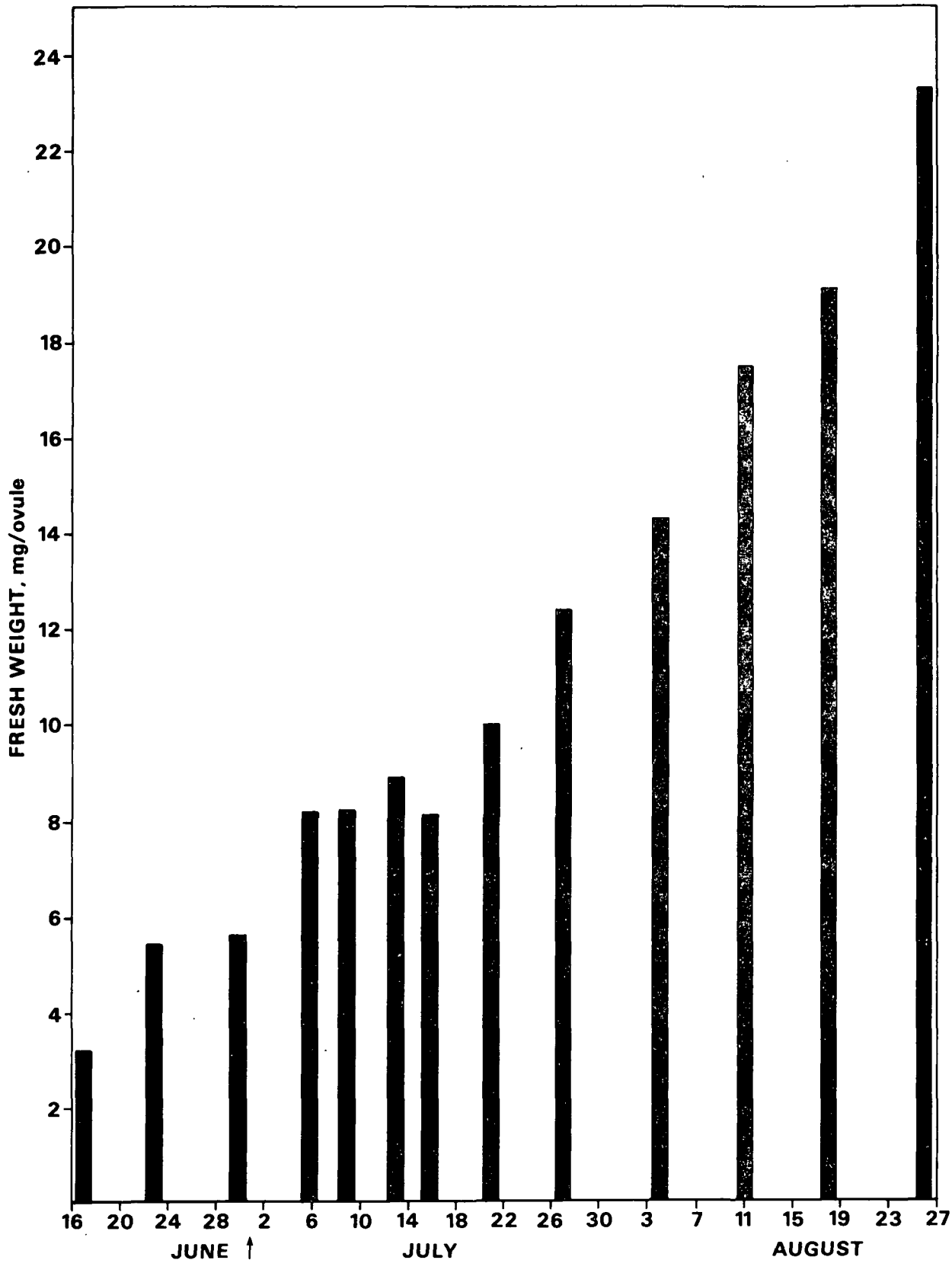

Fig. 2. Changes in white pine ovule fresh weight as a function of development time. The arrow indicates the approximate time of fertilization.